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Original Article

Protective effect against focal cerebral ischemia injury in acute phase of a novel invasive device for regional hypothermia

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Abstract

Background: Systemic hypothermia is considered beneficial to stroke patients. However, many complications ensue. The aim of this study was to evaluate the effect of a new invasive regional cooling device in cerebral ischemic injury in a rat model.
Methods: After a pilot study confirming the efficiency of the cooling device, 15 adult male Sprague–Dawley rats, weighing 300–400 g, were randomly assigned into three groups: cooling device applied at 14°C and at 26°C, and a sham group. Focal cerebral ischemic injury was achieved by electrocauterization of the left middle cerebral artery through craniectomy and temporal occlusion of both common carotid arteries for 3 hours. Within 30 minutes after the end of ischemic injury, the cooling device was inserted into the rat brain through a stereotactic frame to provide regional hypothermia for 2 hours. The rats were sacrificed immediately after the 2-hour regional hypothermia.
Results: Although triphenyltetrazolium chloride staining showed smaller ischemic lesions in both the 26°C and 14°C groups compared to the control group, Fluoro Jade C staining showed no neuroprotective effects in the rostrum cerebral cortex in both groups. However, both triphenyltetrazolium chloride and Fluoro Jade C staining indicated significant beneficial effects in the caudal cerebral cortex in rats with cooling device applied at 26°C compared to the 14°C and control groups.
Conclusion: Our findings indicated that the device can effectively achieve regional hypothermia and could be beneficial for patients with cerebral ischemia during the acute phase.

Keywords: brain ischemia; hypothermia; middle cerebral artery infarction; stroke

1. Introduction

Cerebral ischemic injury is one of the major causes of human stroke.[1] Although tissue plasminogen is currently the only drug approved by the United States Food and Drug Administration, several therapeutic measures, such as neuroprotective reagents, anticoagulants, and thrombolytic drugs, have been widely recognized around the world, with promising effects.[2] However, the narrow recommended 3-hour[3] to 4.5-hour[4] therapeutic window largely confines the treatment options and impairs the prognosis of stroke patients.

Conflict of interest: Dr. Ein-Yiao Shen and Taiwan Advanced Sterilization Technology, Inc. have filed an application for a U.S. patent for the design of the "Tissue Cooling Apparatus", while the Taiwanese patent has been approved (Patent 098139852, Taiwan, R.O.C.). The other authors (P.T.L., Y.P.C., and I.H.L.) declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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In addition to the above-mentioned options, systemic hypothermia has long been known to provide stroke patients certain neuroprotective effects in experimental settings.[5,6] However, whether systemic hypothermia is beneficial or not is still controversial in clinical settings due to several side effects of systemic hypothermia, including arrhythmia, pneumonia, and thrombocytopenia, that may increase morbidity and mortality of the patients and the supportive care burden of health care personnel.[7,8] Accordingly, methods and devices that provide regional hypothermia have been developed to cool through blood vessels,[9−12] nasal cavity,[13−18] meninges[19−21] and the head.[22−27] Unfortunately, the cooling efficiency through brain parenchyma has not been measured in most of those devices, and only a few studies have explored the neuroprotective effects of regional hypothermia.[21,28−31] In addition, the optimal temperature range for regional hypothermia is still controversial.[28,29,32]

This study tested a newly designed invasive device that provides regional hypothermia via direct contact with brain parenchyma at the ischemic center. We explored its cooling efficiency and evaluated its neuroprotective effect in a rat model of permanent middle cerebral artery occlusion.

2. Methods

2.1. Cooling device

Our novel invasive cooling device was designed with a structure of concentric cylinders constituted of a 23-gauge and 18-gauge needle with the tip welded to be sealed. Hence, water could run through the 23-gauge needle and flow out through the 18-gauge needle (Fig. 1A). The hubs of both needles were kept intact to facilitate connection to extension tubes. A digital thermometer was attached to the extension tube close to the entry of water in order to monitor input water temperature (t1, Fig. 1B). The water came from two sets of infusion bags wrapped by an infusion bag pressor that provided the driving force of water at a constant pressure of 300 mmHg.

2.2. Animals

Eighteen adult male Sprague−Dawley rats (BioLasco, Taipei, Taiwan) weighing 300−400 g were used in this study. All rats were housed in 12/12-hour light and dark circadian cycles with free−access to food and water. All management and procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University, Taipei, Taiwan (NTU-99-EL-1).

2.3. Anesthesia and preparation

Anesthesia was induced using 800 mL/min 5% isoflurane (Baxter, Deerfield, IL, USA) mixed with 100% oxygen within an enclosed cage. After intubation with a 16-gauge intravenous catheter, anesthesia was maintained with a small animal ventilator (SAR-830/P; CWE Inc., Ardmore, PA, USA). Surgical areas were shaved and sterilized. In order to monitor the arterial pressure continuously, the right femoral artery was catheterized with a polyethylene tube (PE-50) and connected to a digital blood pressure probe (BP-100; iWorx Systems, Dover, NH, USA). Arterial blood was sampled from the right femoral artery for blood gas analysis prior to and 30 minutes after the common carotid arteries (CCAs) were occluded. In the ischemic study, blood gas was also evaluated 1 hour after the cooling process had started. Anesthetic depth was adjusted to maintain mean arterial pressure between 90 mmHg and 120 mmHg. A digital thermometer (TM-100; iWorx Systems) was placed 3 cm deep into the rectum to monitor the body temperature continuously (214 Data Recorder; iWorx Systems). The body temperature was maintained at 37 ± 0.5°C throughout the experiment by applying alcohol or a heat pad.

2.4. Cooling efficiency

After anesthesia and preparation as described above, three rats were placed in a stereotactic frame. A midline incision and blunt dissection was made to expose the frontal bone, and three holes were drilled by electric drill (Fig. 1C). The cooling center (c, Fig. 1C) was located 6 mm left of the bregma, while two thermometer-probing points were 3 mm caudal and 5 mm left of the bregma (t2, Fig. 1C) and 5 mm rostral and 2 mm left of the bregma (t3, Fig. 1C). After the dura was cut through using a 27-gauge needle, the cooling device was placed 7 mm below the skull surface at the cooling center and digital thermometers were placed 3 mm below the skull surface in the two thermometer-probing points. The temperatures were recorded for 20 minutes, and the final temperatures were marked as static brain temperatures. After the static brain temperature was acquired, 10°C, 14°C, 20°C, and 26°C cold water was perfused through the cooling device serially for 20 minutes, and the final brain temperatures were also recorded at thermometer-probing points t2 and t3 to evaluate the efficiency of the cooling device. The brain was allowed to return to the static brain temperature between each water temperature.

2.5. Brain ischemic model

Fifteen rats were randomly allocated to three groups (14°C, 26°C, and sham control) of five rats each, and were anesthetized and prepared as described above. Permanent middle cerebral artery occlusion (MCAO), which was modified from a previous study,[33] was performed. A ventral midline incision was made to expose the bilateral CCAs and vagosympathetic trunk. After the skin incision, the soft tissue at the surgical site was immersed in 0.5% bupivacaine (Marcaine; AstraZeneca, London, UK) before the CCAs were isolated. Both CCAs were prepared with a snare composed of 6/0 nylon suture and a 1-mm long PE-50 tube for temporary occlusion after the middle cerebral artery was cauterized. To cauterize the middle cerebral artery, the left eyelid was first closed by 6/0 nylon suture and a 1.5-cm incision was made above the zygomatic bone. The left zygomatic bone was removed to expose the temporomandibular junction and the joint capsule, and the ligament was cut. Ventral retraction of the left mandible

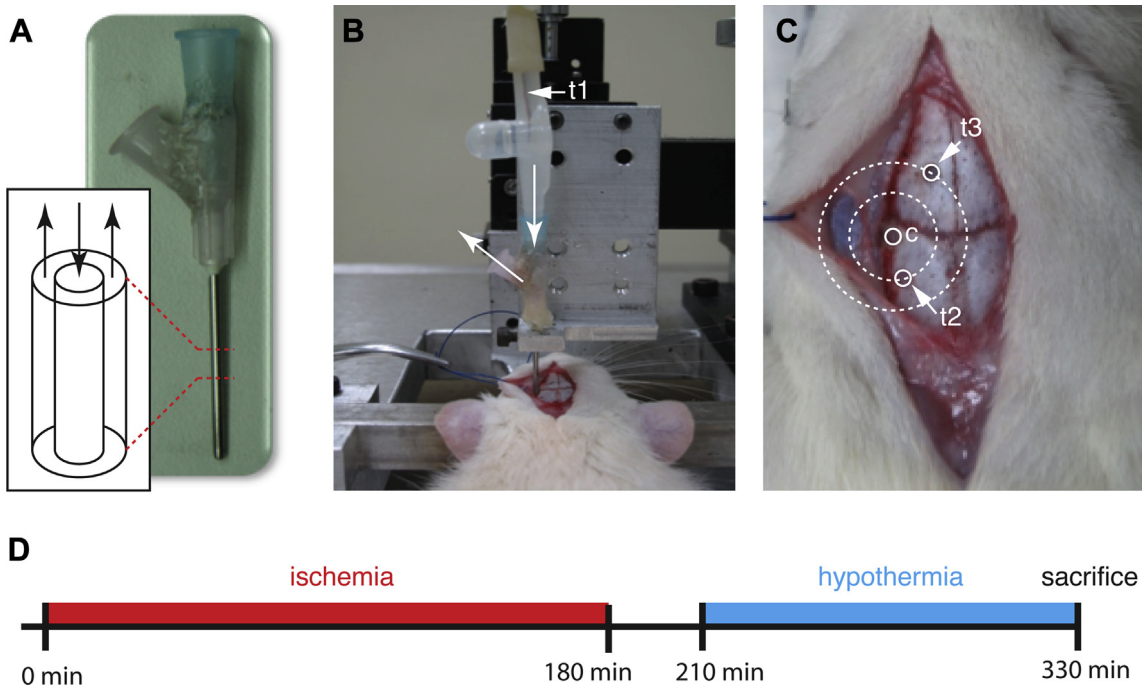

Fig. 1. The representative diagrams demonstrate the designs of the tissue cooling apparatus and the study. (A) The tissue cooling apparatus consisted of two syringe needles to form a concentric cylinder, with the opening of the outer needle blunted and sealed and the inner needle open to the outer needle so that the cooling effect could be conducted by the flushing of precooled water from the inner needle (blue) and the heat could be carried away from the outer needle (pink). (B and C) The cooling center (c in panel C) for the pilot study to assess the cooling efficiency was located 6 mm left of the bregma. The temperature of the cooling water was controlled by a water pump with a temperature controller, while the input temperature (t1 in panel B) and two thermometer-probing points (t2 and t3 in panel C) were monitored. (D) In the brain ischemia experiment, the rat brain was experimentally ischemic for 3 hours and 2 hours hypothermia was started within 30 minutes immediately after the end of the ischemia. The rat brain was then harvested and examined for ischemic damage.

exposed the surface of the temporal bone. A 2-mm hole was drilled under surgical microscopy on the temporal bone 3 mm rostrodorsal to the foramen ovale. The dura was cut using a 27-gauge needle to expose the left middle cerebral artery, which was cauterized by bipolar cautery. To complete the ischemic injury, the skin incision on the head was closed. The CCAs were pulled into the PE-50 tube by stretching both ends of the 6/0 nylon to achieve occlusion for 3 hours (Fig. 1D).

Within 30 minutes after CCA occlusion, the cooling device was set up (Fig. 1D). The CCAs were released and restoration of blood flow was visually confirmed. The rats were placed on a stereotactic frame and a dorsal midline incision over the skull was made to expose the bregma. An opening 1.25 mm in diameter was electrodrilled 3 mm to the left of the bregma, and the dura was cut using a 27-gauge needle. The cooling device was placed 7 mm below the skull surface. Water at 14°C or 26°C was perfused through the cooling device for 2 hours, and a sham operation group without running water was used as a control (Fig. 1D). When cooling was finished, the rats were sacrificed immediately by intracardiac perfusion with overdose of thiamylal sodium and 200 mL cold phosphate-buffered solution (Fig. 1D).

2.6. Histopathological examination

To evaluate ischemic damage, the brain was harvested immediately and cut into eight slices of 2-mm thickness by a brain box. All the slices were immersed in 0.05% 2,3,5-triphenyltetrazolium chloride (TTC; Panreac Quimica, Barcelona, Spain) at 37°C for 30 minutes and fixed in 10% neutral formalin for 24 hours prior to micrography (DM-2500; Leica, Wetzlar, Germany). After all slices were photographed, brain slices of three rats in each group were embedded in paraffin wax and 6-μm-thick sections were cut for further evaluation.

For general morphological changes, hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan) staining by standard method was performed. To evaluate the neuronal degeneration, Fluoro-Jade C (FJC; EMD Millipore Chemicals, Billerica, MA, USA) staining was performed as previously described.[34] The sections were dewaxed by 10 minutes xylene immersion followed by rehydration in 100%, 95%, 80%, and 60% ethanol for 5 minutes each and deionized water for 5 minutes twice. The sections were immersed in 0.06% potassium permanganate for 17 minutes, washed with deionized water, and stained by freshly prepared 0.001% FJC staining solution (0.001% FJC in 0.09% acetic acid) for 30 minutes in the dark. The sections were washed three times in deionized water and dried at 50°C for 20 minutes. The results were documented by fluorescence micrographs (DM-2500; Leica).

2.7. Image analysis

All images were analyzed by computer software ImageJ.[35] For TTC staining, the images of the two hemispheres in all

slices were separated manually. The mean grey values were acquired with black defined as zero and white as 255. The severity of ischemic damage was calculated by dividing the mean grey value of the left hemisphere (ipsilateral to the ischemia) to the right (contralateral to the ischemia), and the resulting value was defined as $G_{L/R}$. Each slice was numerically named from rostral to caudal as slice #1–#8, and hence $G_{L/R1}$ to $G_{L/R8}$ were the $G_{L/R}$ values from corresponding numbered slices. To compare the effect of distance from cooling device, the mean $G_{L/R}$ value of slices #1–#5, mean $G_{L/R1-5}$, was compared to the mean $G_{L/R}$ value of slices #6–#8, mean $G_{L/R\ 6-8}$, while the mean $G_{L/R1-8}$ was the average of all $G_{L/R}$ values of the same brain as the indication of ischemic damage as a whole.

For FJC staining, four independent views at 200× magnification were randomly chosen from the ischemia area of the left hemisphere and the contralateral area of right hemisphere from each brain slice of #2, #3, #6, and #7. The mean grey value of obvious negative area plus three times the standard deviation was used as the threshold to delineate the positive area.[36] For the general protective effect of the affected hemisphere, the average positive area of all four views from each slice in the left hemisphere was divided by the average positive area in the right, and the resulting value was defined as mean $J_{L/R}$. To compare the effects of distance from cooling device, the mean $J_{L/R}$ value of slices #2 and #3, mean $J_{L/R2-3}$, was compared to the value of slices #6 and #7, mean $J_{L/R6-7}$.

2.8. Statistical analysis

Brain temperature and physiological values among the groups were examined by one-way analysis of variance followed by Tukey's test as the *post hoc* analysis. Image analysis of $G_{L/R}$, mean $G_{L/R1-8}$, mean $G_{L/R1-5}$, mean $G_{L/R\ 6-8}$, mean $J_{L/R2-3}$, and mean $J_{L/R6-7}$ were compared by two-way analysis of variance followed by Bonferoni post tests for statistical significance. A p value < 0.05 was regarded as significant. All values were presented as mean ± standard deviation.

3. Results

The design principle of this invasive cooling device is to provide regional hypothermia with the ischemic center as the cooling center so that the device can provide protection covering the area suffering the ischemic damage. It is of interest to evaluate the cooling efficiency and depict the hypothermic area, which in turn suggests the protective area. The static brain temperatures measured after craniectomy were 32.7 ± 0.6°C 3.16 mm from the cooling center (t2) and 33.1 ± 0.4°C 6.40 mm from the cooling center (t3) (Fig. 2, Ctrl). As the input water temperature lowered to 26°C, 20°C, 14°C and 10°C, the temperature at t2 also decreased, to 30.9 ± 0.7°C, 29.1 ± 1.2°C, 27.27 ± 1.1°C, and 26.23 ± 1.0°C, while the temperature at t3 decreased to 32.5 ± 0.4°C, 31.8 ± 0.6°C, 31.2 ± 0.5°C, and 30.9 ± 0.3°C (Fig. 2). Statistically significant differences were detected in every other group at thermometer-probing point t2 (Fig. 2, $p < 0.05$ at Ctrl vs. 20°C and 20°C vs. 10°C; Table S1). At thermometer-probing point t3, the temperature was also decreased as the input temperature lowered, but the differences were not as significant as at thermometer-probing point t2 (Fig. 2, $p < 0.05$ at Ctrl/26°C vs. 14°C/10°C and 20°C vs. 10°C; Table S2). This result indicated that the cooling efficiency decreased significantly as the distance increased and suggested that the hypothermic effect was mainly confined within a relatively small area.

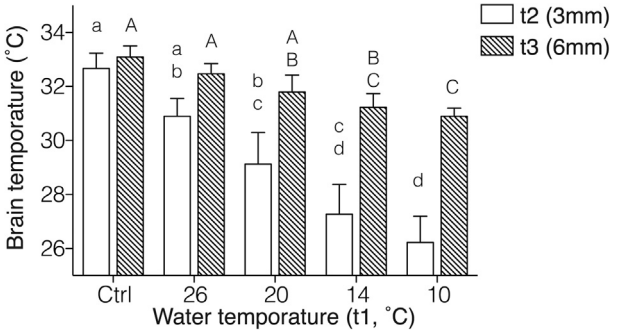

Fig. 2. The tissue cooling apparatus conducted effective hypothermia in a restricted range. The static brain temperature after craniectomy 3.16 mm (t2, open bars) from the cooling center was 32.66 ± 0.33°C, and 33.10 ± 0.23°C 6.40 mm (t3, shaded bars) from the cooling center. Although the brain temperature at t2 significantly decreased as the input water temperature decreased, the brain temperature was not decreased as effectively at t3. The letters above each column indicate the statistical groups, and the data sharing the same letters indicates no significant difference.

Due to the difficulty of maintaining a low temperature of 10°C at the cooling center because of inconsistent measurement of the output temperature (data not shown), we carried out the following experiments with the temperature of 26°C and 14°C as our experimental groups. The physiological measurements, including body weight, body temperature, and blood pH value, arterial oxygen tension (PaO_2) and arterial tension of carbon dioxide ($PaCO_2$), showed no significant difference among the mean values of all three groups (Table 1), suggesting that our device provided strictly regional effects and hence avoided systemic side effects.

The severity of ischemic damage of each brain slice was evaluated by TTC staining (Fig. 3A–C). In our experimental setting, the cooling center was located at the border region between slices #2 and #3. Gross inspection of both of the

Table 1
Mean physiological measurement of the three groups.

		14°C	26°C	Control	p
pH	Pre-ischemic	7.446	7.464	7.478	0.66
	Peri-ischemic	7.404	7.484	7.416	0.31
	Post-ischemic	7.474	7.416	7.395	0.58
$PaCO_2$	Pre-ischemic	39	37.2	34.8	0.71
	Peri-ischemic	40	34.6	39	0.71
	Post-ischemic	32	39.2	37.7	0.28
PaO_2	Pre-ischemic	470.4	410.8	432.8	0.28
	Peri-ischemic	426	451.2	433.4	0.83
	Post-ischemic	467.2	414.8	409.25	0.19
BW (g)		354.2	342.2	333.2	0.49

BW = body weight; $PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension.

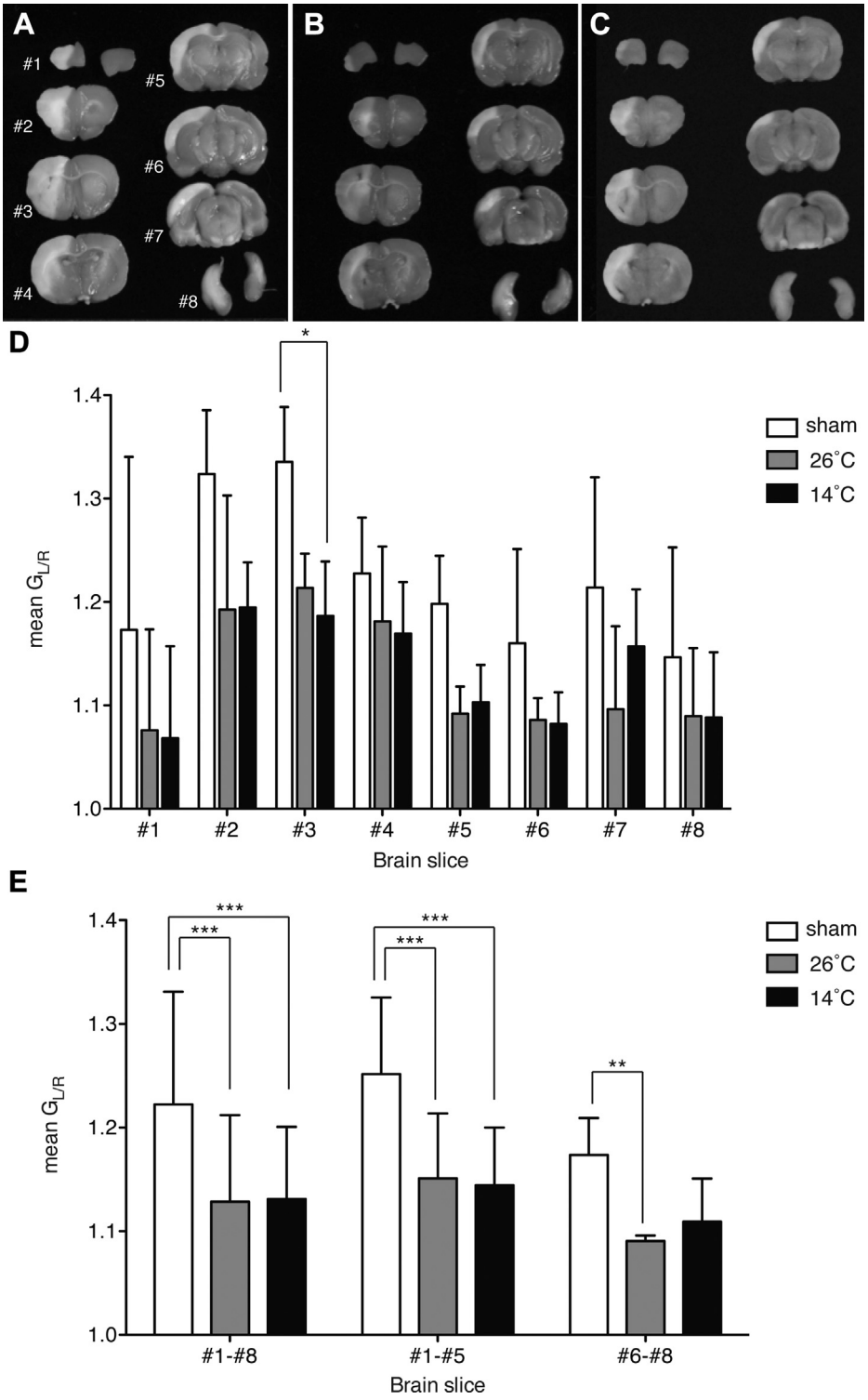

Fig. 3. TTC staining indicated the protective effect of the tissue cooling apparatus against ischemia. (A–C) The brain slices from the sham control group (A), the 26°C hypothermia group (B) and the 14°C hypothermia group (C) were numbered from rostral (#1) to caudal (#8), and the ischemic damage was assessed by TTC staining in which the whitish staining indicate ischemic damage. (D) Image analysis of the TTC staining over the brain slices showed significant difference between the 14°C group and the sham control group at slice #2, and between the hypothermia groups and sham control at slices #3 and #5. (E) The collective profile analysis indicated that both hypothermia groups provided general protection against the ischemia. Although the protection in the rostral region (#1–#5) was equal between the two hypothermia groups, only the 26°C group demonstrated significant protection in the caudal region (#6–#8). *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. TTC = 2,3,5-triphenyltetrazolium chloride.

hypothermic groups showed that the ischemic region, delineated by TTC staining, displayed marked pink areas inside the clear white ischemic regions in contrast to the control group. Moreover, some subjects in the 26°C group showed smaller ischemic region when compared to those in other groups (Fig. 3B). The image analysis detected significantly decreased values of mean $G_{L/R}$ only in slice #3 between the control and 14°C groups (Fig. 3D, $p = 0.033$). However, mean $G_{L/R1-8}$ (Fig. 3E, $p < 0.001$) and mean $G_{L/R1-5}$ (Fig. 3E, $p < 0.001$) of both hypothermic groups were significantly lower than those of the control group, while such differences did not exist between the two hypothermic groups. Nevertheless, the mean $G_{L/R6-8}$ (Fig. 3E, $p = 0.007$) of the 26°C group was significantly lower than that of the other groups, which corresponded to the gross inspection.

Histopathological examination observed morphological changes in all rats, such as condensed perikarya, swollen astrocytes, and less eosinophilic appearance due to the neuropil sponginess (Fig. 4). These findings were in accordance with a previous description of ischemic damage.[37] Moreover, neuropil sponginess was most prominent at the junction between ischemic and relative normal areas (Fig. 4).

To evaluate the effect of neuronal protection, FJC staining was performed. Many neurons with condensed perikarya were positively stained (Fig. 5A). Moreover, gross inspection revealed lower positive density in the 26°C group in contrast to the other groups (Fig. 5A). Image analysis indicated that the 26°C group contained significantly lower positive area intensity at brain slices #6 and #7 when compared with the control group (Fig. 5B, $p = 0.0009$), suggesting that this treatment provides extensive neuronal protection at the ischemic border region, which is consistent with the observation in TTC staining. Surprisingly, the 14°C group not only showed no significant difference to the control group in caudal region (slices #6 and #7), but also displayed a significantly higher positive value than the control group ($p = 0.0158$) and the 26°C group ($p = 0.0006$) in the rostral region (slices #2 and #3; Fig. 5B).

4. Discussion

In this study, we described a new invasive device that provides regional hypothermia by direct contact with the ischemic center. For evaluating the cooling efficiency, the static brain temperature in our study was 32–33°C, lower than the 36°C of previous reports.[19,21,38] This could be due to the difference in surgical approach, as craniectomy was performed in our experimental setting for the placement of temperature probes and consequently promoted temperature radiation from the open skull. Accordingly, craniectomy with a larger area covering the ischemic region might be an option not only for decompression but also for a certain extent of regional hypothermia at the superficial cortex.[39] Furthermore, the documented temperature differences (26°C: −1.7°C and −0.7°C; 20°C: −3.5°C and −1.3°C; 14°C: −5.4°C and −1.9°C; 10°C: −6.3°C and −2.2°C at 3.16 mm and 6.40 mm distances, respectively) in the superficial cortex in our study suggested a lower cooling efficiency compared to direct liquid perfusion (20°C: −3–−4°C; 14°C: −9°C)[19,21] and cooling coil placed between the temporal muscle and skull (31°C: −5°C; 26°C: −10°C).[38] Our result clearly showed that the cooling effect was confined within a relatively small area, implying that multiple devices might be required in clinical applications. Although the true therapeutic effect requires further evaluation, one advantage to our design is that the hypothermia could reach a deeper layer of the cortex compared to the superficial cooling methods. Further revisions, such as accelerated flow rate, substitution with better conductive material, and increasing contact area by remodeling the device shape are pending to improve the cooling efficiency and effective area.

It is generally believed that ischemia/reperfusion injury results in dynamic lesion progression. However, the duration and extent of the progression varies depending on the evaluation methods, the model of MCAO and different rat strains.[40–44] A previous study using diffusion and perfusion magnetic resonance imaging showed that the cerebral blood-flow-derived lesion volumes in Sprague–Dawley rats remained constant

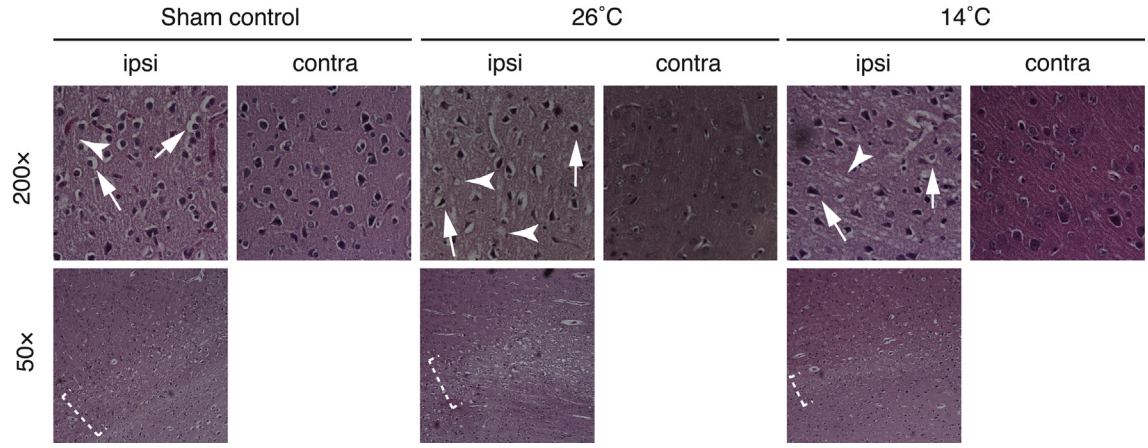

Fig. 4. The histopathology examination showed typical morphological changes of brain ischemia. In the higher-power magnification (200×, upper panel), typical morphological changes of ischemic damage including condensed perikarya (arrows) and neuropil sponginess (arrowheads) could be found in the brain tissue on the ischemic side (ipsi) compared to the undamaged contralateral brain (contra). In the lower-power magnification (50×, lower panel), the most severe neuropil sponginess could be found at the junction of ischemia and relative normal area.

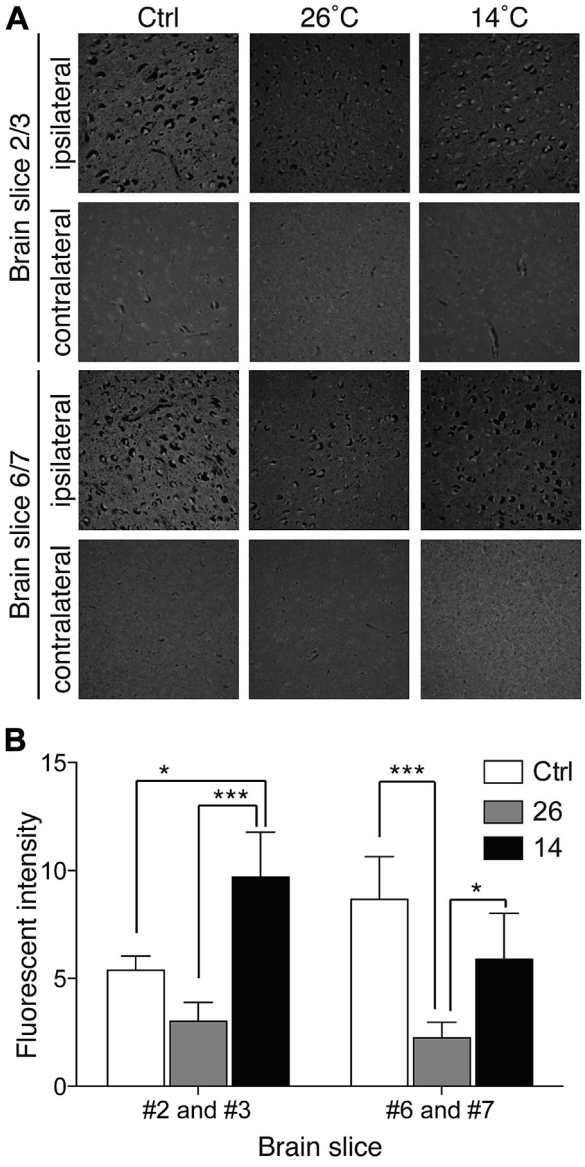

Fig. 5. Hypothermia at 26°C exerted the best neural protection against ischemia. (A) Fluoro-Jade C staining was applied to the brain samples, and neural damage was observed under green fluorescent microscopy (200×). (B) Quantitative analysis of the fluorescence micrographs indicated an adverse effect of hypothermia of 14°C around the cooling center, while 26°C hypothermia exerted a neural protective effect on the caudal brain that was more remote to the cooling center. *$p < 0.05$. ***$p < 0.001$.

45 minutes after permanent MCAO and were highly correlated with the TTC-defined infarct volume at 24 hours.[40] Moreover, the lesion volume derived from the apparent diffusion coefficient stopped growing after 3 hours in this rat strain.[40] In the present study, the experimental animals were euthanized immediately after 2 hours regional hypothermia with a total of 5.5 hours of MCAO. It is reasonable to expect that an ischemic lesion volume correlating with the final infarct would result.

We used TTC to evaluate the ischemic damage to the neural tissue because it was considered a reliable method to determine the lesion area of cerebral ischemia.[45] TTC is a water-soluble salt that is converted to formazan, a red and lipid-soluble substance, by mitochondrial respiratory chain. When there is tissue ischemia and in turn dysfunction of the mitochondrial respiratory chain, TTC staining presents a white color compared to the red color in the healthy tissues as TTC is converted into formazan by healthy mitochondria. In this work, instead of a clear-margined white area, we observed pinkish staining with a white background scattered in the ischemic area in the hypothermic groups (Fig. 3A–C), suggesting certain retention of the intact mitochondrial respiratory chain within the hypothermic area.

To further evaluate the neuroprotective effect of regional hypothermia, we performed FJC staining to identify degenerated neurons. Although this method cannot differentiate necrosis from apoptosis and the exact working mechanism remains unknown,[46] a previous study showed that degenerated neurons could be identified 6 hours after ischemic injury in a transient MCAO rat model.[47] In accordance with TTC staining, FJC staining detected significant neuronal protection in the caudal region (brain slices #6 and #7) only in the 26°C group (Fig. 5B). By contrast, while TTC staining suggested that both hypothermic groups provided obvious protective effects against ischemia in the rostral region (Fig. 3D and E), no significant neuronal protection was detected in this region according to FJC staining (Fig. 5B). Furthermore, the neuronal degeneration was even more severe in the 14°C group as FJC-positive area intensity was significantly higher than in the other two groups (Fig. 5B), implying that the neuronal damages other than ischemia, such as cold shock, could be introduced. Consistent with our findings, a recent study demonstrated deep hypothermia at 17°C resulted in increased cell death in the hippocampus, with decreased expression of hypothermia-inducible genes such as RBM3.[48] Although the optimal temperature for the best protective effect in therapeutic hypothermia may vary in different cooling methods, this might explain our results that 26°C hypothermia provided better overall protection, while severe neuronal degeneration near the cooling center was observed in the 14°C group.

To mimic the conditions of stroke patients in clinical practice, it is reasonable to initiate hypothermia after an ischemic period, however, only a few studies on therapeutic hypothermia have been done in this way.[49–52] Overall, 1 hour has been suggested as the maximum delay for hypothermia to start after the end of ischemia, since initiating hypothermia after that time not only universally provides no protective effects, but also potentiates an even larger ischemic area than the control.[49] To evaluate the therapeutic effect of our new device critically, bilateral CCA occlusion was maintained for 3 hours, which was longer than in previous studies,[49–52] and therefore the ischemic injury should have been more profound than in previous studies. Taken together, the observation of neuronal protective effect against ischemic damage in the caudal region of the 26°C group under a longer period of ischemia with delayed onset of cooling indicated the therapeutic potential of regional hypothermia.

In conclusion, we have described a newly designed invasive device that can provide effective regional hypothermia in the brain. Although the optimal therapeutic temperature is not described in this study and requires further research, the

neuronal protective effect against ischemia in the caudal region in the 26°C group provides a good hint to start. Our findings in this study suggest that, although more remains to be elucidated, therapeutic hypothermia holds potential for further research and application.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2014.07.009.

References

1. Simon RP, Greenberg DA, Aminoff MJ. *Clinical neurology*. 7th ed. New York: Lange Medical Books/McGraw-Hill; 2009.
2. Suwanwela N, Koroshetz WJ. Acute ischemic stroke: overview of recent therapeutic developments. *Annu Rev Med* 2007;**58**:89−106.
3. Group TNIoNDaSr-PSS. Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. *N Engl J Med* 1995;**333**:1581−7.
4. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008;**359**:1317−29.
5. van der Worp HB, Sena ES, Donnan GA, Howells DW, Macleod MR. Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis. *Brain* 2007;**130**:3063−74.
6. Yanamoto H, Nagata I, Niitsu Y, Zhang Z, Xue JH, Sakai N, et al. Prolonged mild hypothermia therapy protects the brain against permanent focal ischemia. *Stroke* 2001;**32**:232−9.
7. Den Hertog HM, van der Worp HB, Tseng MC, Dippel DW. Cooling therapy for acute stroke. *Cochrane Database Syst Rev* 2009. CD001247.
8. Sarkar S, Barks JD. Systemic complications and hypothermia. *Semin Fetal Neonatal Med* 2010;**15**:270−5.
9. Allers M, Boris-Moller F, Lunderquist A, Wieloch T. A new method of selective, rapid cooling of the brain: an experimental study. *Cardiovasc Intervent Radiol* 2006;**29**:260−3.
10. Ding Y, Li J, Rafols JA, Phillis JW, Diaz FG. Prereperfusion saline infusion into ischemic territory reduces inflammatory injury after transient middle cerebral artery occlusion in rats. *Stroke* 2002;**33**:2492−8.
11. Wei G, Hartings JA, Yang X, Tortella FC, Lu XC. Extraluminal cooling of bilateral common carotid arteries as a method to achieve selective brain cooling for neuroprotection. *J Neurotrauma* 2008;**25**:549−59.
12. Schwartz AE, Stone JG, Finck AD, Sandhu AA, Mongero LB, Adams DC, et al. Isolated cerebral hypothermia by single carotid artery perfusion of extracorporeally cooled blood in baboons. *Neurosurgery* 1996;**39**:577−81.
13. Busch HJ, Eichwede F, Fodisch M, Taccone FS, Wobker G, Schwab T, et al. Safety and feasibility of nasopharyngeal evaporative cooling in the emergency department setting in survivors of cardiac arrest. *Resuscitation* 2010;**81**:943−9.
14. Covaciu L, Allers M, Enblad P, Lunderquist A, Wieloch T, Rubertsson S. Intranasal selective brain cooling in pigs. *Resuscitation* 2008;**76**:83−8.
15. Dohi K, Jimbo H, Abe T, Aruga T. Positive selective brain cooling method: a novel, simple, and selective nasopharyngeal brain cooling method. *Acta Neurochir Suppl* 2006;**96**:409−12.
16. Einer-Jensen N, Khorooshi MH, Petersen MB, Svendsen P. Rapid brain cooling in intubated pigs through nasal flushing with oxygen: prevention of brain hyperthermia. *Acta Vet Scand* 2001;**42**:459−64.
17. Hagioka S, Takeda Y, Takata K, Morita K. Nasopharyngeal cooling selectively and rapidly decreases brain temperature and attenuates neuronal damage, even if initiated at the onset of cardiopulmonary resuscitation in rats. *Crit Care Med* 2003;**31**:2502−8.
18. Natale JA, D'Alecy LG. Protection from cerebral ischemia by brain cooling without reduced lactate accumulation in dogs. *Stroke* 1989;**20**:770−7.
19. Cheng H, Shi J, Zhang L, Zhang Q, Yin H, Wang L. Epidural cooling for selective brain hypothermia in porcine model. *Acta Neurochir* 2006;**148**:559−64.
20. Forte LV, Peluso CM, Prandini MN, Godoy R, Rojas SS. Regional cooling for reducing brain temperature and intracranial pressure. *Arq Neuropsiquiatr* 2009;**67**:480−7.
21. Noguchi Y, Nishio S, Kawauchi M, Asari S, Ohmoto T. A new method of inducing selective brain hypothermia with saline perfusion into the subdural space: effects on transient cerebral ischemia in cats. *Acta Med Okayama* 2002;**56**:279−86.
22. Battin MR, Penrice J, Gunn TR, Gunn AJ. Treatment of term infants with head cooling and mild systemic hypothermia (35.0 degrees C and 34.5 degrees C) after perinatal asphyxia. *Pediatrics* 2003;**111**:244−51.
23. Diao C, Zhu L. Temperature distribution and blood perfusion response in rat brain during selective brain cooling. *Med Phys* 2006;**33**:2565−73.
24. Tooley JR, Eagle RC, Satas S, Thoresen M. Significant head cooling can be achieved while maintaining normothermia in the newborn piglet. *Arch Dis Child Fetal Neonatal Ed* 2005;**90**:F262−6.
25. Wang H, Olivero W, Lanzino G, Elkins W, Rose J, Honings D, et al. Rapid and selective cerebral hypothermia achieved using a cooling helmet. *J Neurosurg* 2004;**100**:272−7.
26. Clark DL, Colbourne F. A simple method to induce focal brain hypothermia in rats. *J Cereb Blood Flow Metab* 2007;**27**:115−22.
27. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. *Exp Neurol* 2007;**208**:277−84.
28. Barone FC, Feuerstein GZ, White RF. Brain cooling during transient focal ischemia provides complete neuroprotection. *Neurosci Biobehav Rev* 1997;**21**:31−44.
29. Ding Y, Li J, Luan X, Lai Q, McAllister 2nd JP, Phillis JW, et al. Local saline infusion into ischemic territory induces regional brain cooling and neuroprotection in rats with transient middle cerebral artery occlusion. *Neurosurgery* 2004;**54**:956−64.
30. Lo EH, Steinberg GK. Effects of hypothermia on evoked potentials, magnetic resonance imaging, and blood flow in focal ischemia in rabbits. *Stroke* 1992;**23**:889−93.
31. Zhang L, Cheng H, Shi J, Chen J. Focal epidural cooling reduces the infarction volume of permanent middle cerebral artery occlusion in swine. *Surg Neurol* 2007;**67**:117−21.
32. Kollmar R, Blank T, Han JL, Georgiadis D, Schwab S. Different degrees of hypothermia after experimental stroke: short- and long-term outcome. *Stroke* 2007;**38**:1585−9.
33. Yanamoto H, Nagata I, Hashimoto N, Kikuchi H. Three-vessel occlusion using a micro-clip for the proximal left middle cerebral artery produces a reliable neocortical infarct in rats. *Brain Res Brain Res Protoc* 1998;**3**:209−20.
34. Kanthasamy AG, Yun RJ, Nguyen B, Truong DD. Effect of riluzole on the neurological and neuropathological changes in an animal model of cardiac arrest-induced movement disorder. *J Pharmacol Exp Ther* 1999;**288**:1340−8.
35. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;**9**:671−5.
36. Zhang SJ, Zou M, Lu L, Lau D, Ditzel DA, Delucinge-Vivier C, et al. Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PloS Genet* 2009;**5**:e1000604.
37. Garcia JH, Yoshida Y, Chen H, Li Y, Zhang ZG, Lian J, et al. Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol* 1993;**142**:623−35.

38. Clark DL, Penner M, Wowk S, Orellana-Jordan I, Colbourne F. Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat. *Exp Neurol* 2009;**220**:391−9.
39. Stone JG, Goodman RR, Baker KZ, Baker CJ, Solomon RA. Direct intraoperative measurement of human brain temperature. *Neurosurgery* 1997;**41**:20−4.
40. Bardutzky J, Shen Q, Henninger N, Bouley J, Duong TQ, Fisher M. Differences in ischemic lesion evolution in different rat strains using diffusion and perfusion imaging. *Stroke* 2005;**36**:2000−5.
41. Garcia JH, Liu KF, Ho KL. Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. *Stroke* 1995;**26**:636−42.
42. Murphy BD, Chen X, Lee TY. Serial changes in CT cerebral blood volume and flow after 4 hours of middle cerebral occlusion in an animal model of embolic cerebral ischemia. *AJNR Am J Neuroradiol* 2007;**28**: 743−9.
43. Nedergaard M. Neuronal injury in the infarct border: a neuropathological study in the rat. *Acta Neuropathol* 1987;**73**:267−74.
44. Saita K, Chen M, Spratt NJ, Porritt MJ, Liberatore GT, Read SJ, et al. Imaging the ischemic penumbra with 18F-fluoromisonidazole in a rat model of ischemic stroke. *Stroke* 2004;**35**:975−80.
45. Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke* 1986;**17**:1304−8.
46. Schmued LC, Stowers CC, Scallet AC, Xu L. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. *Brain Res* 2005;**1035**:24−31.
47. Butler TL, Kassed CA, Sanberg PR, Willing AE, Pennypacker KR. Neurodegeneration in the rat hippocampus and striatum after middle cerebral artery occlusion. *Brain Res* 2002;**929**:252−60.
48. Tong G, Endersfelder S, Rosenthal LM, Wollersheim S, Sauer IM, Buhrer C, et al. Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices. *Brain Res* 2013;**1504**:74−84.
49. Baker CJ, Onesti ST, Solomon RA. Reduction by delayed hypothermia of cerebral infarction following middle cerebral artery occlusion in the rat: a time-course study. *J Neurosurg* 1992;**77**:438−44.
50. Doerfler A, Schwab S, Hoffmann TT, Engelhorn T, Forsting M. Combination of decompressive craniectomy and mild hypothermia ameliorates infarction volume after permanent focal ischemia in rats. *Stroke* 2001;**32**: 2675−81.
51. Kader A, Brisman MH, Maraire N, Huh JT, Solomon RA. The effect of mild hypothermia on permanent focal ischemia in the rat. *Neurosurgery* 1992;**31**:1056−61.
52. Moyer DJ, Welsh FA, Zager EL. Spontaneous cerebral hypothermia diminishes focal infarction in rat brain. *Stroke* 1992;**23**:1812−6.